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13. ABSTRACT ***(Maximum 200 Words)***

During the first year of funding, we accomplished the synthesis of three of the four protected **Linker-Drug** conjugates of doxorubicin and 5-fluorouracil (5-FU) proposed in the original application (Task 1). We have also successfully tested the cyclization activation process of one type of **Linker-Drug** conjugate of 5-FU (Task 2). Our results established chemically the feasibility of using carbamoyl-5-fluorouracil conjugates as potential prodrugs for the treatment of cancer such as advanced prostate cancer. Some of this work was presented at the 217th national meeting of the American Chemical Society in Anaheim, California. Efforts are now focused on the synthesis of the remaining **Linker-Drug** conjugate of 5-FU, selective reduction of the nitro and azido group in the conjugates of doxorubicin, and the kinetic analysis of the cyclization activation process.

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Introduction

During the first year of funding, we accomplished the synthesis of three of the four protected **Linker-Drug** conjugates of doxorubicin and 5-fluorouracil (5-FU) proposed in the original application (Task 1). We have also successfully tested the cyclization activation process of one **Linker-Drug** conjugate of 5-FU (Task 2). Some of this work was presented at the 217th national meeting of the American Chemical Society in Anaheim, California. Efforts are now focused on the synthesis of the remaining **Linker-Drug** conjugate of 5-FU, selective reduction of the nitro and azido group in the conjugates of doxorubicin, and the kinetic analysis of the cyclization activation process.

Annual Report Body

In our original application, we proposed to accomplish task 1 and part of task 2 during the first year of funding. Task 1 was to chemically synthesize and characterize four protected **Linker-Drug** conjugates of doxorubicin and 5-FU, two anticancer agents that have shown some therapeutic response in the treatment of advanced prostate cancer (months 1-10). Of the four proposed, we have successfully synthesized and characterized three. Difficulty in the synthesis of fourth conjugate may be attributed to the inherent less nucleophilic nature of the aromatic amine.

Task 2 was to determine the kinetics of cyclization-activation process of the Linker-Drug conjugates in buffered aqueous solutions (months 11-14). We have successfully tested one of the **Linker-Drug** conjugates synthesized after selective reduction of the protecting nitro group.

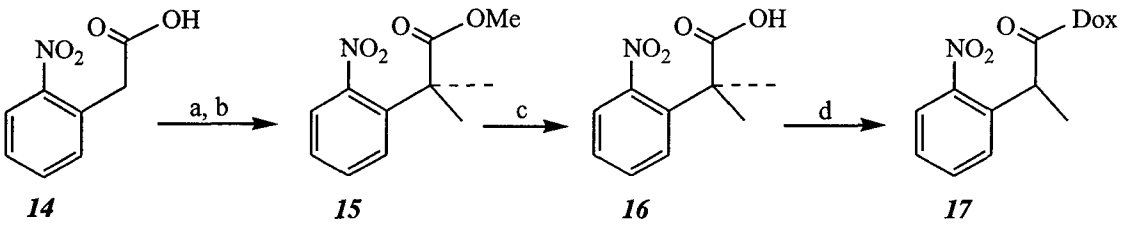
For the convenience of reviewers, I have used the same compound numbers in this progress report as in the original application. To do this, it was necessary to use suffixes such as ***25b*** and ***25c*** to designate intermediates and analogues.

1) Synthesis of protected Linker-Drug conjugates of doxorubicin and 5-FU

Because of the potential facile cyclization of **Linkers** having a free amino group to form the corresponding lactam or cyclic urea, we used synthetic strategies that mask the amino group as an inert nitro or azido group. Reduction to the corresponding amino group will take place only when needed.

*Synthesis of 2-(2-nitrophenyl)-2-methyl-propionic acid-Doxorubicin conjugate (**17**).* As shown in Scheme 1, we started the synthesis with *2*-nitrophenylacetic acid (***14***). Esterification of ***14*** using thionyl chloride in methanol followed by α,α-dialkylation using sodium hydride and methyl iodide in the presence of catalytic amount of 18-crown-6 gave the α,α-dimethyl analogue ***15*** in quantitative yield. Sodium hydroxide-mediated hydrolysis converted ester ***15*** to its corresponding acid ***16*** in 93% yield.

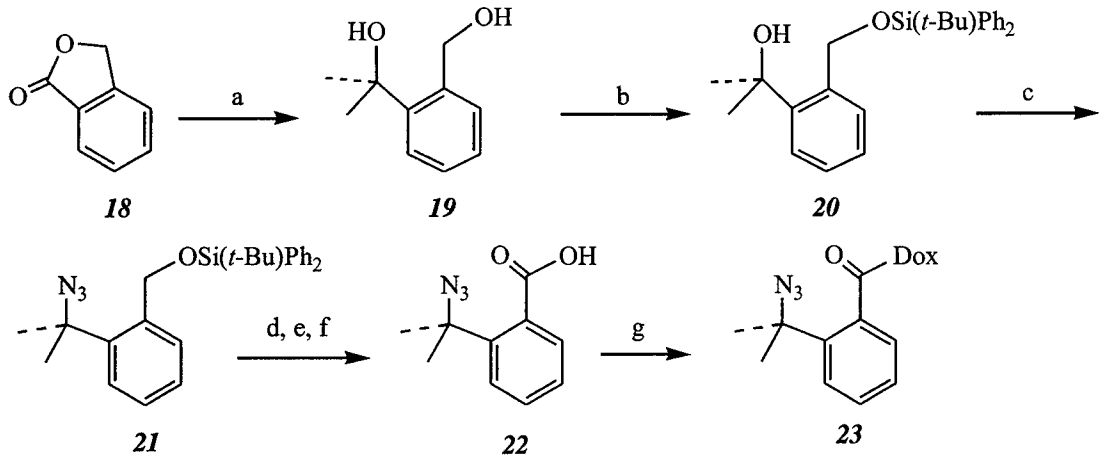
Scheme 1.



14 ***15*** ***16*** ***17***

a) $SOCl_2$/MeOH, 100%; b) MeI/NaH, 18-crown-6, 100%; c) 2 N NaOH/MeOH, reflux, 6 hr, 93.0%; d) HBTU/DIEA, Dox·HCl, 40.0%

Scheme 2.



a) MeMgBr followed by H_2O, 100%; b) 1.1 eq. t-$BuPh_2SiCl$/imidazole/THF, 92.4%;
c) NaN_3/TFA/CH_2Cl_2, 73%; d) TBAF/THF, 73.4%; e) PDC/DMF, 100%;
f) $NaClO_2$, NaH_2PO_4, 64%; g) HBTU/DIEA/DMF, Dox·HCl, 62.6%

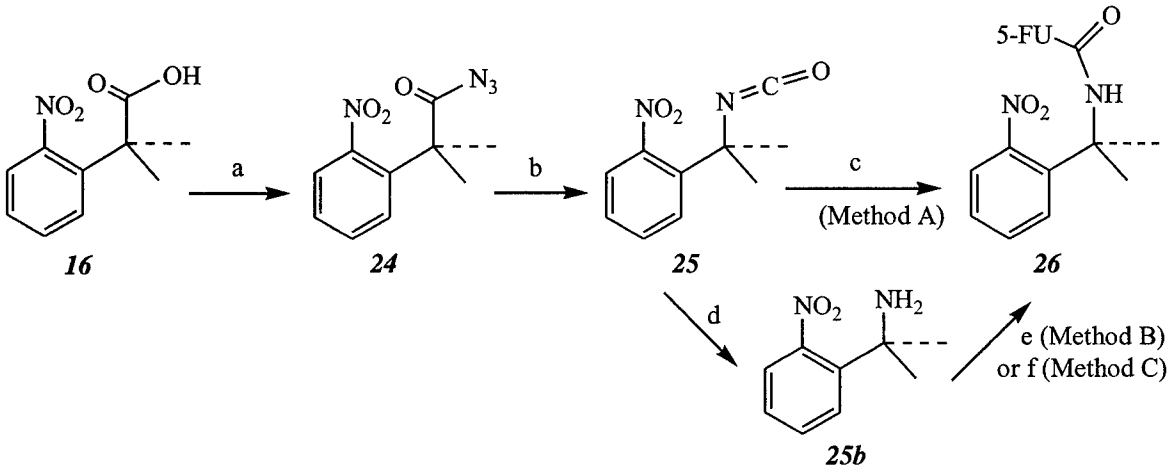
Coupling of the acid ***16*** to the amino group of doxorubicin was accomplished using its HOBt activated ester to give the protected **Linker-Drug** conjugate ***17*** in 40% yield.

Synthesis of 2-(1-azidoisopropyl)-benzoic acid-Doxorubicin conjugate (***23***). As shown in Scheme 2, Grignard reaction of the commercially available phthalide (***18***) with methyl magnesium bromide gave diol ***19*** in quantitative yield. The primary alcohol in ***19*** was selectively protected by t-butyldiphenylsilyl group to form the silyl ether compound ***20*** in 92.4% yield. The secondary hydroxyl group in compound ***20*** was replaced by an azido group using sodium azide and TFA giving the azido compound ***21*** in 73% yield. Deprotection of the silyl ether ***21*** by fluoride ion and subsequent two-step oxidation of primary alcohol to the corresponding carboxylic acid afforded compound ***22*** in 47.0% yield. Coupling of the carboxylic acid to the drug doxorubicin led to the protected **Linker-Drug** conjugate ***23*** in 62.6% yield.

*Synthesis of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (**26**) and N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (**26b**).* Scheme 3 shows the synthesis of nitro-**Linker-Drug** conjugate ***26*** of 5-FU starting from compound ***16***. The acid ***16*** was first converted to the acyl azide followed by trapping of the Curtius rearrangement intermediate ***25*** with 5-FU to give N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-FU (***26***) in an overall yield of 3.6% (Method A). The strong refluxing condition and low yield in the last step of this method prompted us to explore an alternative route. Acid hydrolysis of the Curtius rearrangement intermediate ***25*** gave the free primary amine ***25b***. Final condensation with 5-FU could then be accomplished by reaction of the amine with diphosgene followed by coupling of the amine-chlorofomate with 5-FU sodium salt (Method B) or reaction of the free amine with N^1-chloroformyl-5-FU (Method C). We found that method C offers a very mild reaction condition as well as a better yield.

To study the effect of the two methyl groups on the rate of cyclization activation process, we also synthesized N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***26b***), an analogue without the two methyl groups. As shown in Scheme 4, 2-nitrophenylacetic acid was converted to 2-nitrobenzylamine in 76.1% yield by treatment with sodium azide and sulfuric acid in chloroform at 50 °C for 1.5 h followed by neutralization with sodium hydroxide. Coupling with 5-FU by using N^1-chloroformyl-5-FU afforded the desired N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***26b***) in 34.2% yield.

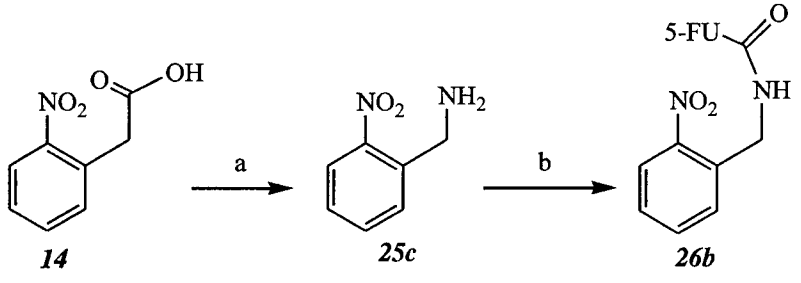
Scheme 3.



16 ***24*** ***25*** ***26*** ***25b***

a) $ClCO_2Et/Et_3N$ followed by NaN_3, 31.3%; b) reflux, toluene, 2 hr, 79.5%; c) 5-FU/Et_3N, toluene, reflux, 18 hr, 14.3%; d) HCl, 32.0%; e) diphosgene/C, followed by 5-FUNa 16.7%; f) 5-FU-COCl, rt, 50.0%

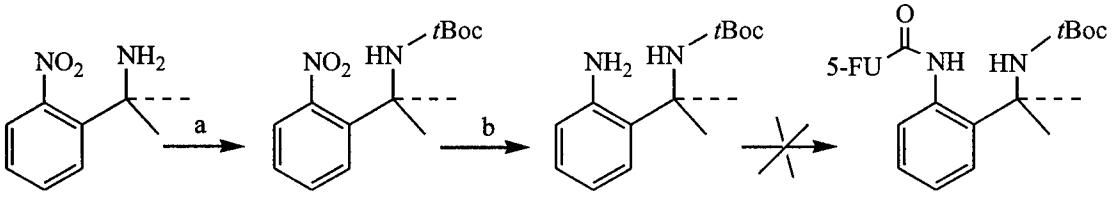
Scheme 4.



14 ***25c*** ***26b***

a) H_2SO_4, NaN_3, $CHCl_3$, 50 °C, 1.5 hr, followed by NaOH, 76.1%; b) 5-FU-COCl, rt, 34.2%

Scheme 5.

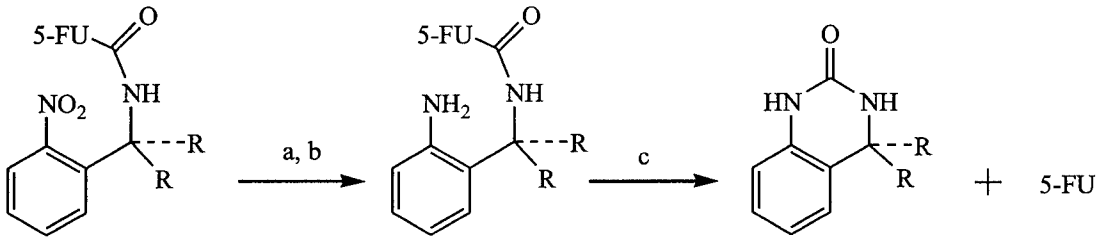


25c ***27b*** ***28b*** ***29b***

a) $(Boc)_2O$, 88.2%; b) H_2, 10% Pd/C, 70.9%;

Synthesis of N^1-[2-(1-azidoisopropyl)phenylcarbamoyl]-5-fluorouracil ***(29)***. We are having trouble synthesizing this compound as originally proposed because of the inherent difficulty of coupling an aromatic amine with N^1-chloroformyl-5-FU. As shown in Scheme 5, reaction of compound ***28b***, where the azido group is replaced by a *t*-Boc-amino group, with N^1-chloroformyl-5-FU failed to give the desired conjugate ***29b***. This has been reported by Ozaki and colleagues [1]. We will continue to explore other methods of synthesis in the second year of support.

Scheme 6.



26 R = Me
26b R = H

10 R = Me
10b R = H

11 R = Me
11b R = H

a) H_2, 10% Pd/C; b) $NaBH_4$, 10% Pd/C; c) Phosphate buffer, pH 7.4, 37 °C

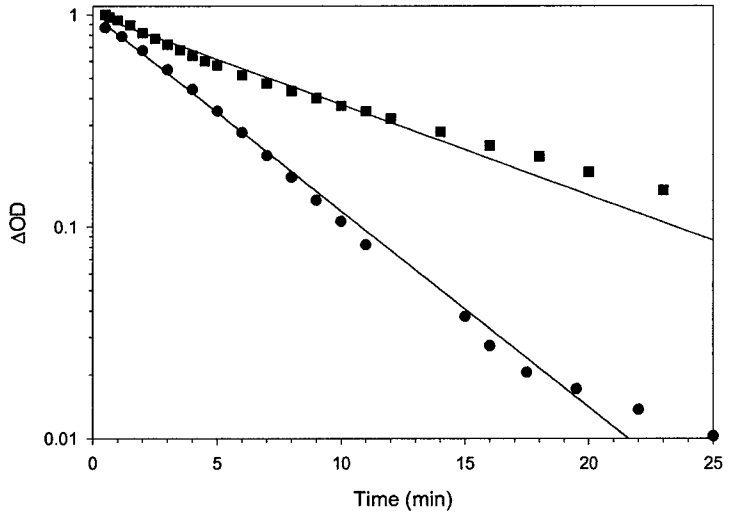


Figure 1. The kinetics of cyclization reaction of 2-aminophenylalkylcarbamoyl-FU as monitored by following the decrease in UV absorption at 235 nm corresponding to the disappearance of the starting amino starting material. Shown were the best fit calculated for the cyclization of 2-aminophenylisopropylcarbamoyl-FU (■——■) and 2-aminobenzylcarbamoyl-FU (●——●).

2) Reduction of the N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (*26*) and N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (*26b*) and the kinetic analysis of the cyclization-activation process

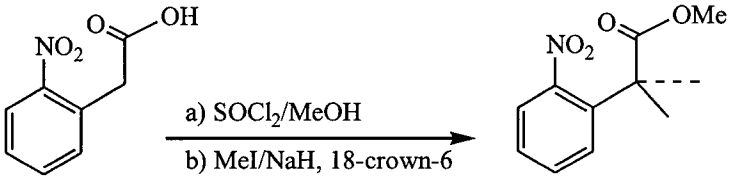
For the doxorubicin-conjugates ***17*** and ***23***, we have yet to find a reduction condition that is selective for the nitro/azido group over the quinone moiety in doxorubicin. We found that the methods suggested in the original proposal also affect the quinone functionality. Other methods will be evaluated in the selective reduction of nitro and azido groups in these conjugates.

The FU conjugates ***26*** and ***26b*** synthesized were successfully reduced to the corresponding amino group by hydrogenation and sodium borohydride over 10% Pd/C (Scheme 6). While cyclization occurs to a greater extent under the condition of sodium borohydride, hydrogenation over 10% Pd/C gave predominantly the amine intermediates ***10*** and ***10b***. The cyclization activation process was then monitored by UV/Vis spectrometry in 50 mM sodium phosphate buffer, pH 7.4 at 37 °C (Figure 1). As

discussed in our original application, the two methyl groups in compound ***10*** were introduced to limit the rotational freedom of the carbamoyl side chain relative to the amino group so as to increase the rate of cyclization activation process [2]. To our surprise, we found that N^1-[1-(2-aminophenyl)isopropylcarbamoyl]-5-fluorouracil ***10***, a conjugate with the two methyl groups, has a half life of 7.1 min in pH 7.4 phosphate buffer at 37 °C while N^1-(2-aminobenzylcarbamoyl)-5-fluorouracil ***10b***, a conjugate without the two methyl groups, has even a shorter half life of 3.3 min under the same incubation conditions (Figure 1). Apparently, this system of ours works differently from the literature system of 2-aminophenylacetates [3]. The products of the cyclization reaction were characterized by analytical HPLC, NMR, and MS and confirmed by the use of authentic samples.

3) Experimental Section

*Synthesis of 2-(2-nitrophenyl)-2-methylpropionic acid methyl ester (**15**).*

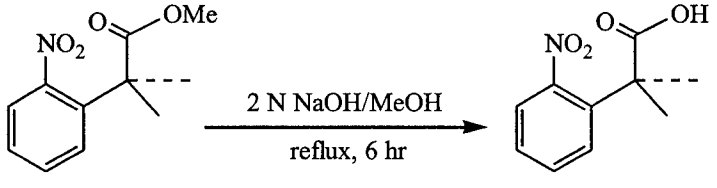


14 ***15***

To a solution of 2-nitrophenylacetic acid ***14*** (40 g, 0.22 mol) in 240mL methanol was added with stirring $SOCl_2$ (36 mL) over 30 min while maintaining the temperature at 0-4 °C. The reaction mixture was stirred at 0 °C for 1 h and at room temperature for 18 h. After removal of solvent, the residue was dissolved with ethyl acetate, washed with water, and dried over $MgSO_4$. Removal of ethyl acetate under reduced pressure gave the corresponding methyl ester (45 g, 100%). MS (FAB, NBA) *m/z* 196.2 (MH^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 3.72 (s, 3H, OCH_3) 4.04 (s, 2H, CH_2), 7.38-8.14 (m, 4H, Ph).

The methyl ester (19.5 g, 100 mmol), MeI (14.3 mL, 250 mmol, 2.5eq) and 18-crown-6 (6.6 g, 25 mmol, 0.25 eq) was dissolved in 130 mL of DMF and stirred at -4-0 °C. A small amount of sodium hydride (60% in oil) was added slowly until the color suddenly turned to blue; then NaH (9.2g, 230 mmol, 2.3eq,) was added with stirring over 40 min while maintaining the temperature at 0-4 °C. The reaction mixture gradually turned to a green then yellow slurry after standing overnight. The reaction mixture was then diluted with ethyl acetate, washed with 1 N HCl, 1 N $KHCO_3$, and brine, and dried over $MgSO_4$. Removal of ethyl acetate afforded desired 2-(2-nitrophenyl)-2-methylpropionic acid methyl ester ***15*** (26.9 g, 100%). MS (FAB, NBA) *m/z* 224.2 (MH^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.68 (s, 6H, CH_3), 3.65 (s, 3H, CH_3), 7.26-7.89 (m, 4H, Ph).

*Synthesis of 2-(2-nitrophenyl)-2-methylpropionic acid (**16**).*

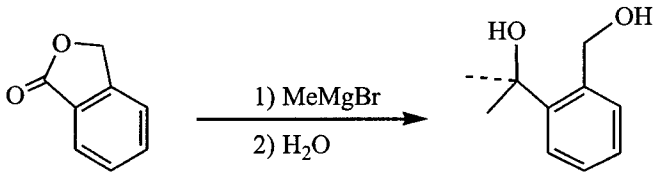


15 ***16***

2-(2-Nitrophenyl)-2-methylpropionic acid methyl ester ***15*** (8 g, 36 mmol) was refluxed in 216 mL of 1 N NaOH (6 eq) methanol/water (1 : 1) solution for 6 h. After evaporation of methanol, the aqueous solution was acidified with 1 N HCl to pH 2 and extracted with ethyl acetate. The ethyl acetate phase was then washed with brine, and dried over $MgSO_4$. Removal of ethyl acetate gave 2-(2-nitrophenyl)-2-methylpropionic acid ***16*** (5.5 g, 93%) as a yellow solid in. IR (KBr) cm^{-1} 2900 (br), 2600,

1700; MS (EI) *m/z* 164.3 (M^+-COOH, 3.6%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.71 (s, 6H, CH_3), 7.61-7.99 (m, 4H, Ph).

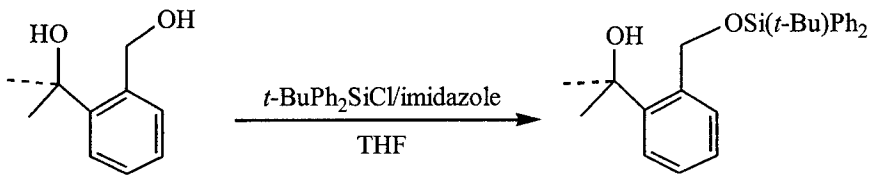
*Synthesis of 2-(1-hydroxylisopropyl)-benzyl alcohol (**19**) [4].*



18 ***19***

A solution of phthalide (20 g, 149 mmol) in 100 mL CH_2Cl_2 was added through a dropping funnel into a 100 mL solution of 3 M MeMgBr in Et_2O at 0-5 °C (ice-salt bath) under N_2. The reaction mixture was stirred at 5-10 °C for 1 hour, and then diluted with ethyl acetate. After washing with aqueous NH_4Cl and brine, it was dried over $MgSO_4$. Removal of organic solvents gave the desired product ***19*** (25.2 g, 100%) as an oil. MS (EI) *m/z* 148 (M^+-H_2O, 83.4%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.67 (s, 6H, CH_3), 3.40-3.60 (b, 2H, OH), 4.81 (s, 2H, $HOCH_2$), 7.20-7.29 (m, 4H, Ph).

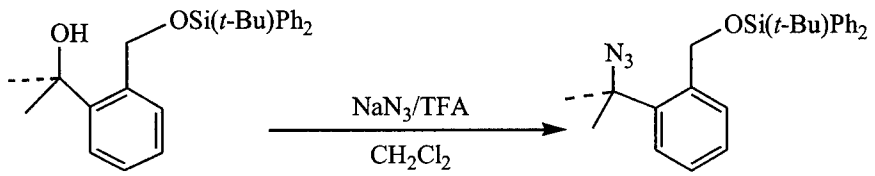
*Synthesis of 2-(1-hydroxylisopropyl)-benzyloxy-t-butyl-diphenylsilane (**20**) [5].*



19 ***20***

Flame-dried 3-necked flask was charged with diol ***19*** (10 g, 60.2 mmol), *t*-butyldiphenylsilyl chloride (18.1 g, 66.2 mmol, 1.1 eq) and 60 mL of tetrahydrofuran under N_2. The mixture was then cooled to 0 °C. A solution of DMAP (30 mg, 0.004 eq), imidazole(20.5 g, 300.8 mmol, 5 eq) in 90 mL THF was added slowly. After the reaction mixture was stirred for 1 h, it was diluted with ethyl acetate, washed with 5% $KHSO_4$ solution and brine, dried over $MgSO_4$. Removal of solvent afforded the desired product ***20*** (22.5 g, 92.4%) as an oil. MS (FAB, NBA) *m/z* 405 (MH^+); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.05 (s, 9H, *t*-Bu), 1.63 (s, 6H, CH_3), 5.01 (s, 2H, OCH_2), 7.12-7.68 (m, 14H, Ph).

*Synthesis of 2-(1-azidoisopropyl)-benzyloxy-t-butyl-diphenylsilane (**21**) [6].*

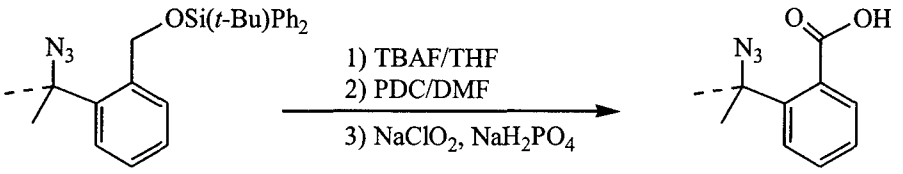


20 ***21***

To a solution of trifluoroacetic acid (15mL, 14 eq) in 120 mL of CH_2Cl_2 was added sodium azide (8 g, 8 eq) at 0 °C. After 10 min at 0 °C, a solution of 2-(1-hydroxylisopropyl)-benzyloxy-*t*-butyl-diphenylsilane ***20*** (5.9 g, 14.6 mmol) in 120 mL of CH_2Cl_2 was added dropwise over 15 min. The reaction mixture was allowed to warm up to room temperature and stirred for 48 h. It was then diluted with CH_2Cl_2, washed with sat. $KHCO_3$ and brine, then dried over Na_2SO_4. After evaporation of solvent under reduced pressure, the residue was separated by flash column chromatography to give the desired product ***21*** (3.4 g, 73% after recovering 1.73 g starting material) as an oil. IR (KBr) cm^{-1} 2100 (-N_3); MS

(FAB, NBA) *m/z* 386 (M^+-HN_3, 2.8%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.08 (s, 9H, *t*-Bu), 1.53 (s, 6H, CH_3), 5.05 (s, 2H, OCH_2), 7.30-7.90 (m, 14H, Ph).

*Synthesis of 2-(1-azidoisopropyl)-benzoic acid (**22**) [5].*



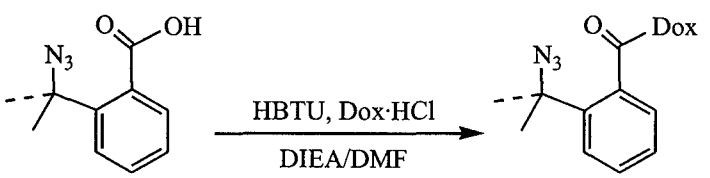
21 ***22***

To a solution of 2-(1-azidoisopropyl)-benzyloxy-t-butyl-diphenylsilane ***21*** (918 mg, 2.1 mmol) in 5 mL of THF was added 3.4 mL of 1 M TBAF (1.6 eq) in THF. The reaction mixture was stirred at room temperature for 2 h before it was diluted with ethyl acetate, washed with 5% $NaHSO_4$ and brine, dried over Na_2SO_4. After removal of solvent, the residue was separated by flash column chromatography to give 2-(1-azidoisopropyl)-benzyl alcohol (300 mg, 73.4%) as an oil. IR (KBr) cm^{-1} 3320 (br, -OH), 2100 (-N_3); MS (EI) *m/z* 149.2 (M^+-N_3, 3.2%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.75 (s, 6H, CH_3), 2.4 (br, 1H, OH), 4.92 (s, 2H, OCH_2), 7.26-7.55 (m, 4H, Ph).

A suspension of 2-(1-azidoisopropyl)-benzyl alcohol (900 mg, 4.7 mmol) and PDC (6.7 g, 3.8 eq) in 20 mL DMF was stirred at room temperature for 4.5 h. The reaction mixture was filtered through a pad of celite, washed with water and brine until no color was present in the organic phase, and then dried over $MgSO_4$. Removal of solvent gave 2-(1-azidoisopropyl)-benzaldehyde as an oil in quantitative yield. IR (KBr): cm^{-1} 2100 (-N_3), 1670 (-CHO); MS (FAB, NBA) *m/z* 147.2 (M^+-N_3, 4.9%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.80 (s, 6H, CH_3), 7.43-7.96 (m, 4H, Ph), 10.90 (s, 1H, CHO)

To a solution of 2-(1-azidoisopropyl)-benzaldehyde (900 mg, 4.7 mmol) in 50 mL of *t*-butanol and 12 mL of 2-methyl-2-butene was added dropwise with stirring a solution of 2.0 g of $NaClO_2$ (5 eq) and 2.3 g of $NaH_2PO_4 \cdot H_2O$ in 20 mL water. Stirring was continued for 2 h at room temperature before the reaction mixture was diluted with water and washed with ether. The aqueous phase was acidified to pH 2 with 1 N HCl solution and extracted with ethyl acetate. The ethyl acetate extract was dried over Na_2SO_4. Removal of solvent under vacuum, the residue was subjected to flash column chromatography to give 2-(1-azidoisopropyl)-benzoic acid ***22*** (620mg, 64%) as a solid. IR (KBr) cm^{-1} 2900(br, -COOH), 2100 (-N_3), 1650 (-COOH); MS (FAB, NBA) *m/z* 206.2 (MH^+, 11.2%); ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.79 (s, 6H, CH_3), 7.32-7.53 (m, 4H, Ph), 8.6 (br, 1H, COOH).

*Synthesis of 2-(1-azidoisopropyl)-benzoic acid-Doxorubicin conjugate (**23**).*

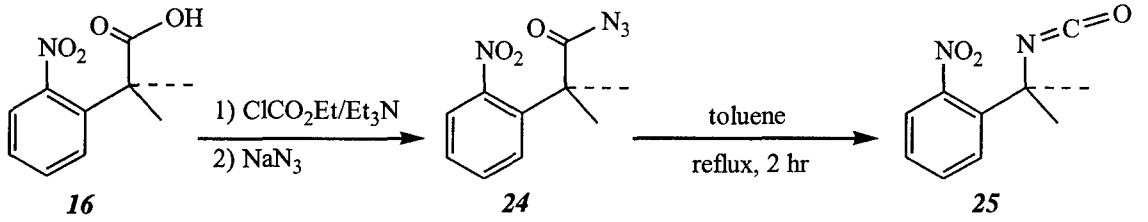


22 ***23***

2-(1-Azidoisopropyl)-benzoic acid (12 mg, 0.059 mmol) and HBTU (23 mg, 0.059 mmol) were dried under vacuum for 1 h before dry DMF was introduced under N_2. After the addition of DIEA (15 μL, 0.059 mmol), the reaction mixture was stirred at room temperature for 15 min followed by the addition of a solution of Dox·HCl (34 mg, 0.059 mmol) and DIEA (15 μL, 0.059 mmol) in 0.5 mL DMF. The reaction mixture was stirred at room temperature for 1 h, diluted with ethyl acetate, washed with 1 N HCl, sat. $NaHCO_3$ and brine, and dried over Na_2SO_4. After removal of solvent under vacuum, the residue was subjected to flash column chromatography to obtain the desired doxorubicin conjugate ***23*** (27 mg,

62.6%) as a red solid. TLC ($CH_3OH/CHCl_3$, 1 : 6) R_f 0.4; IR (KBr) cm^{-1} 3400, 2900, 2100; ^1H NMR (300 MHz, $CDCl_3$), δ 1.33 (d, J = 6.6 Hz, 3H), 1.70 (s, 3H, CH_3), 1.74 (s, 3H, CH_3), 1.90-1.96 (dd, J = 4.2, 13.8 Hz, 1H), 1.98-2.04 (dd, J = 5.4, 14.0 Hz, 1H), 2.22 (d, J = 4.2 Hz, 1H), 2.40 (d, J = 14.3 Hz, 1H), 3.01 (s, 1H), 3.08 (s, 1H), 3.85 (s, 1H), 4.09 (s, 3H), 4.24 (q, J = 6.7 Hz, 1H), 4.30-4.40 (m, 1H), 4.79 (d, J = 4.8 Hz, 2H), 5.31 (s, 1H), 5.56 (d, J = 3.3 Hz, 2H), 6.05 (d, J = 8.1 Hz, 1H), 7.24-8.06 (m, 7H, Ph).

*Synthesis of 2-(2-nitrophenyl)-2-methylpropionyl azide (**24**)* and *1-(2-nitrophenyl)-isopropyl isocyanate (**25**) [7].*

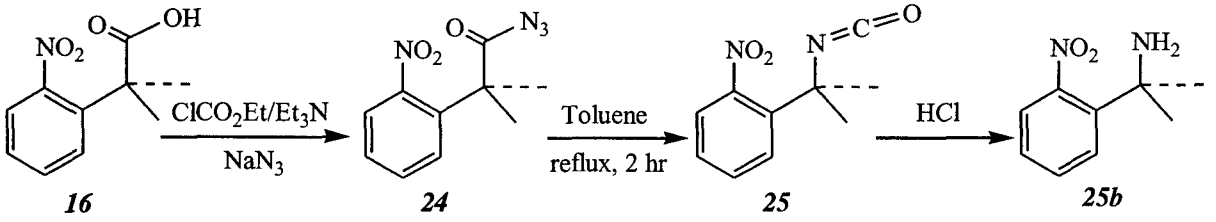


16 ***24*** ***25***

2-(2-Nitrophenyl)-2-methylpropionic acid ***16*** (1.2 g, 6 mmol) was dissolved in 15 mL of dry acetone, to which was added slowly triethylamine (0.92 mL, 1.1 eq) under N_2. The solution was then cooled and kept at -5-0 °C when $ClCO_2Et$ (0.51 mL, 1.1 eq) was added slowly. After stirring for an additional 15 min, NaN_3 (780 mg, 2 eq) in 3 mL water was added slowly over 10 min at -5-0 °C. The stirring was continued for another 30 min at 0 °C before the reaction mixture was poured into cold water and extracted with methylene chloride. The organic phase was washed with brine and dried over Na_2SO_4. Removal of methylene chloride gave the desired acyl azide product ***24*** (400 mg, 31.3%) as an oil after recovering 60 mg starting material. IR (KBr) cm^{-1} 2140, 1700; MS (FAB) *m/z* 235.1(MH^+, 22.2%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.65 (s, 6H, CH_3), 7.46-7.99(m, 4H, Ph).

The acyl azide ***24*** (100 mg, 0.43 mmol) was dissolved in 2 mL of toluene and refluxed for 2 h. The residue after evaporation of solvent was subjected to flash column chromatography on silica gel to give the corresponding isocyanate ***25*** (70 mg, 79.5%) as an oil. IR (KBr) cm^{-1} 2260 (-N=C=O); MS (FAB) *m/z* 205.1 (M^+-1, 3.5%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.87 (s, 6H, CH_3), 7.41-7.52 (m, 4H, Ph).

*One-pot synthesis of 2-nitrophenylisopropylamine (**25b**) from 2-(2-nitrophenyl)-2-methylpropionic acid (**16**) [7].*

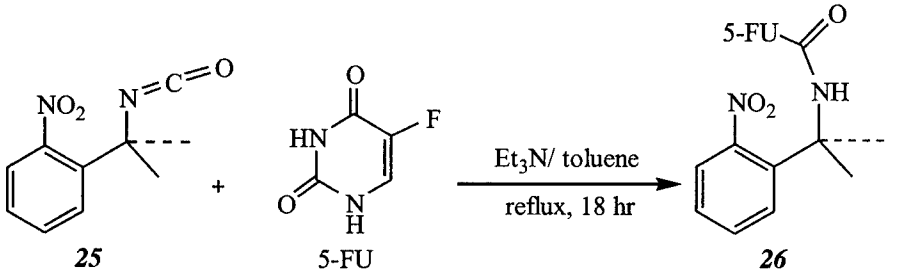


16 ***24*** ***25*** ***25b***

To a solution of 2-(2-nitrophenyl)-2-methylpropionic acid ***16*** (1.25 g, 6 mmol) in 10 mL of acetone was added slowly with stirring 0.92mL (1.1eq) of Et_3N. The mixture was cooled to -5-0 °C before $ClCO_2Et$ (0.63 mL, 1.1 eq) in 2 mL of acetone was added slowly. The reaction mixture was stirred for an additional 15 min at -5-0 °C before a solution of NaN_3 (780 mg, 2eq) in 3 mL water was added slowly. After stirring was continued for another 30 min at -5-0 °C, the reaction mixture was poured into 25 mL cold water and extracted with toluene (2x25 mL). The organic phase was dried over $MgSO_4$ (150 mg acid was recovered from aqueous phase). The toluene extract was transferred to a one necked round bottomed flask equipped with a reflux condenser; the stirred solution was heated cautiously under reflux for 1 h on an electric bath. Toluene was then removed at 50 °C with a rotavap; the flask containing the residual was again fitted with reflux condenser, the oil was stirred and cooled in an ice bath before 10 mL

of 8 N HCl was added. The cooling bath was removed and the stirred mixture was gradually heated under reflux for 10 min. Then evacuated and warmed in a bath at 50 °C for 10 min; 10 mL ice water was added to the flask while cooled in an ice bath, 30 mL of 3 N NaOH solution was added slowly to adjust the pH to 12. Ethyl acetate extraction followed by washing with brine, drying over $MgSO_4$, solvent evaporation gave the desired product 2-nitrophenylisopropylamine ***25b*** (300 mg, 32%) after flash column chromatography. IR (KBr) cm^{-1} 3300, 2950, 1500, 1350; MS (FAB) *m/z* 181.1 (MH^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 1.58 (s, 6H, CH_3), 1.65 (s, 1H, NH), 1.70 (s, 1H, NH), 7.28-7.54 (m, 4H, Ph).

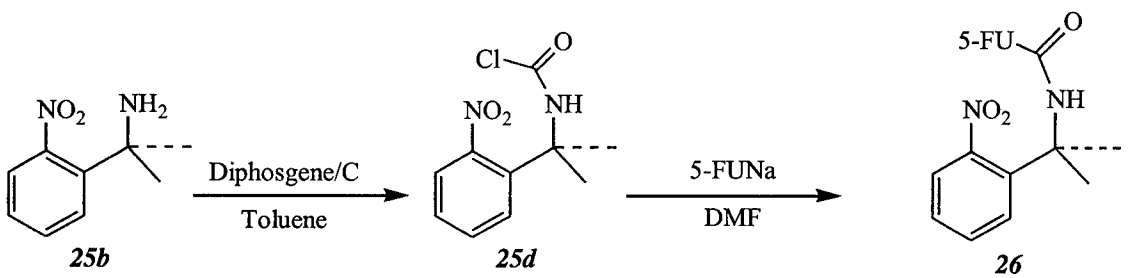
*Synthesis of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (**26**).*
Method A [1]



25 5-FU ***26***

Isocyanate ***25*** (150 mg, 0.7 mmol) and 5-FU (95 mg, 0.7 mmol, 1 eq) were dissolved in 3.5 mL of toluene. After the addition of 0.1 mL of Et_3N, the reaction mixture was refluxed for 18 h. The residue after evaporation of solvent was subjected to flash column chromatography on silica gel to obtain the desired 5-FU conjugate ***26*** (35 mg, 14.3%) as a solid. TLC (EtOAc/PE=1:1) $R_f = 0.5$; MS (FAB) *m/z* 337.1(MH^+, 12.4%); HRMS (FAB, NBA) $C_{14}H_{14}FN_4O_5$ (MH^+) calcd 337.0948, found 337.0950; ^1H NMR (300 MHz, $CDCl_3$) δ 1.88 (s, 6H, CH_3), 7.26-7.54 (m, 4H, Ph), 8.32 (d, $J = 7.2$ Hz, 1H), 9.47 (s, 1H).

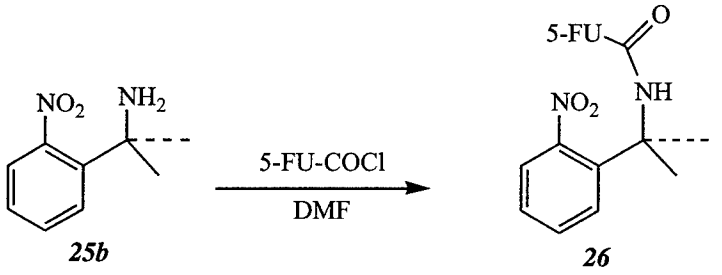
Method B [8]



25b ***25d*** ***26***

2-Nitrophenylisopropylamine ***25b*** (32 mg, 0.2 mmol) and 5 mg of activated charcoal in 1 mL anhydrous toluene were stirred under N_2 at 0 °C. 50 μL of diphosgene (2 eq) was added and the reaction mixture was stirred at room temperature for 18 h. N_2 was bubbled through the reaction mixture to remove excess phosgene; after filtration and CH_2Cl_2 wash, the solvent was evaporated. The residue and 30 mg of 5-FUNa (1 eq) were dried under vacuum for 1 h before 1 mL of anhydrous DMF was introduced under N_2. Stirring was continued at room temperature for 20 h. The reaction mixture was then diluted with ethyl acetate, washed with water and brine, dried over Na_2SO_4. After removal of solvent, the residue was separated using flash column chromatography on silica gel to give the desired 5-FU conjugate ***26*** (10 mg, 16.7%) as a solid.

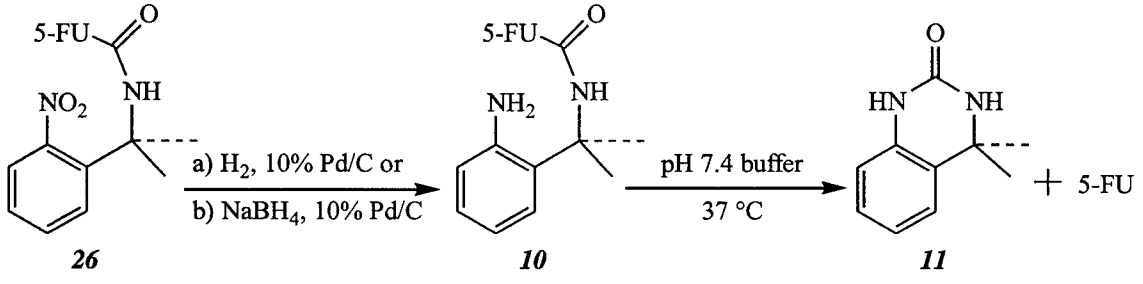
Method C [9]



25b ***26***

5-FU (20 mg, 0.154 mmol) and 10 mg of active charcoal were dried under vacuum for 1 h. 2 mL of pyridine was introduced under argon and mixture was cooled down to 0 °C. Diphosgene (40 μL, 0.308 mmol) was added with stirring. After 2 h, N_2 was bubbled through to remove excess phosgene. After removal of charcoal, the filtrate containing *N*^{1}-chloroformyl-5-FU was added directly to 2-nitrophenylisopropylamine ***25b*** (20 mg, 0.111 mmol). The reaction mixture was stirred at room temperature for 18 h before solvent was removed by rotavap. The residue was dissolved in acetone and the pyridinium salt was removed by filtration. Flash column chromatography gave the desired 5-FU conjugate ***26*** (23 mg, 50%).

Reduction of N^1*-[1-(2-nitrophenyl)-isopropylcarbamoyl]-5-fluorouracil* ***(26)****, kinetic analysis of cyclization activation process and identification of cyclization activation products.*



26 ***10*** ***11***

Hydrogenation. *N*^{1}-[1-(2-nitrophenyl)-isopropylcarbamoyl]-5-fluorouracil ***26*** (10 mg, 0.03 mmol) was dissolved in 0.6 mL of methanol. After the addition of 2 mg of 10% Pd/C, hydrogen was introduced through a balloon and the reduction was monitored by HPLC and TLC.

For kinetic analysis, the reaction mixture was centrifuged after 30 min of reduction and an aliquot of the supernatant was added to pre-warmed 50 mM sodium phosphate buffer (pH 7.4, 37 °C) to give a total volume of 1 mL in the cuvet. The cyclization reaction was then followed by monitoring the change in absorbance at 235 nm, which is the absorption maximum for the intermediate aromatic amine ***10***. The data was then fitted to first order decay kinetics to calculate the first order kinetic constant and the half life of the cyclization activation process.

For the identification of products after cyclization, the reaction was allowed to stand at room temperature for 48 h. At the end of reaction, the catalyst was removed by filtration and solvent by evaporation. The residue was washed with methylene chloride to give 5-FU (2.3 mg, 59.4%) as a white solid. The methylene chloride solution subjected flash column chromatography to give the cyclic urea product ***11*** (2.8 mg, 53%).

$NaBH_4$ reduction. To a solution of *N*^{1}-[1-(2-nitrophenyl)-isopropylcarbamoyl]-5-fluorouracil ***26*** (10 mg, 0.03 mmol) in 1 mL of methanol was added 1 mg of 10% Pd/C catalyst and 8 mg of $NaBH_4$ in 0.5 mL of H_2O under N_2. After 30 min at room temperature, the catalyst was removed by filtration and solvent by evaporation. The residue was taken up by ethyl acetate, washed with brine, dried over

Na_2SO_4. Both the aqueous phase and the organic phase were analyzed by HPLC and TLC. 5-FU was found in aqueous phase and the cyclic urea ***11*** was found in the organic phase. The cyclization reaction was found to be much faster under this reduction condition. Thus, $NaBH_4$ reduction is not suitable for the kinetic analysis of the cyclization activation process.

TLC (acetone/PE = 1 : 1) R_f 0.2 (FU), 0.5 (cyclic urea product ***11***), 0.9 (protected conjugate ***26***); HPLC analysis was performed on a C_{18} reversed-phase column (150 x 4.6 mm) using first isocratic elution of 1.6% acetonitrile for 5 min followed by a gradient elution of 1.6% to 42% acetonitrile in 15 min and final isocratic 42% acetonitrile for additional 5 min at a flow rate of 1 ml/min and detection wavelength of both 220 and 280 nm. The retention times for nitro conjugate ***9***, amino intermediate ***10***, 5-FU and cyclic urea product ***11*** were 28.3, 22.6, 4.59 and 20.48 min, respectively.

Spectra data for the cyclic urea product ***11***: MS (FAB, NBA) *m/z* 177.2 (MH^+, 28%); HRMS (FAB, NBA) $C_{10}H_{13}N_2O$ (MH^+) calcd 177.1028, found 177.1028; ^1H NMR (300 MHz, CD_3OD) δ 1.55 (s, 6H, CH_3), 6.80-7.60 (m, 4H, Ph).

Spectra data for 5-FU: MS (EI) *m/z* 130.1(M^+, 100%); ^1H NMR (300 MHz, $CDCl_3$) δ 7.59 (s, 1H). The HPLC characteristics were also confirmed by comparison with standard samples.

Key Research Accomplishments

1) Synthesis of protected Linker-Drug conjugates of doxorubicin and 5-FU

- Synthesis of 2-(2-nitrophenyl)-2-methyl-propionic acid-Doxorubicin conjugate (***17***).
- Synthesis of 2-(1-azidoisopropyl)-benzoic acid-Doxorubicin conjugate (***23***).
- Synthesis of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (***26***)
- Synthesis of N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***26b***)

2) Selective reduction and the kinetic analysis of the cyclization-activation process

- Selective reduction of N^1-[1-(2-nitrophenyl)isopropylcarbamoyl]-5-fluorouracil (***26***)
- Selective reduction of N^1-(2-nitrobenzylcarbamoyl)-5-fluorouracil (***26b***)
- Kinetic analysis of the cyclization-activation process of N^1-[1-(2-aminophenyl)isopropylcarbamoyl]-5-fluorouracil (***10***)
- Kinetic analysis of the cyclization-activation process of N^1-(2-aminobenzylcarbamoyl)-5-fluorouracil (***10b***)

Reportable Outcomes

Some of this work was presented at the 217th national meeting of the American Chemical Society in Anaheim, California. A copy of the poster is included in the appendices. I expect to publish some of the research results in at least two scientific papers in the coming year of support.

Conclusions

Three of the four protected **Linker-Drug** conjugates were successfully synthesized during the first year of funding. We have also tested successfully the cyclization activation process for one conjugate of 5-fluorouracil after selective reduction. This result established chemically the feasibility of using carbamoyl-5-fluorouracil conjugates as potential prodrugs. Efforts are focused in our laboratory to synthesize the remaining **Linker-Drug** conjugate and test the cyclization activation process of other **linker-drug** conjugates synthesized. We are on schedule and no major changes are needed in future years of support. We expect to complete the rest of the projects within the next 18 months.

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Appendices

1. Copy of the poster presented at the 217th national meeting of the American Chemical Society in Anaheim, California, March 21-25, 1999.

Synthesis and Biomimetic Reductive Activation of Potential Prodrugs of 5-FU and FUDR

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ABSTRACT

5-Fluorouracil (5-FU) and 5-fluoro-2'-deoxyuridine (FUDR) are two fluoropyrimidines used for the treatment of a variety of tumors. They suffer from a number of drawbacks, which include lack of tumor-selectivity, high toxicity, rapid blood clearance, and extensive metabolism to both active and inactive degradation products. To improve oral bioavailability and tumor-selectivity, 5'-[2-(2-nitrophenyl)-2-methylpropionyl]-2'-deoxy-5-fluorouridine and 1-[2-(2-nitrophenyl)-2-methylethylcarbamoyl]-5-fluorouracil were synthesized as potential prodrugs of FUDR and 5-FU to target hypoxic tumor cells or be activated by a nitroreductase. The release of the active drug, FUDR or 5-FU, from the potential prodrugs was found to be very fast after conversion of the nitro group to the amino group. The synthesis and biomimetic reductive activation of the potential prodrugs will be presented.

INTRODUCTION

The major problem with chemotherapy in the treatment of cancer is its lack of selectivity. Most of the drugs used in the clinic are toxic to healthy tissues as well as cancerous cells. 5-Fluorouracil (5-FU, ***1***) and 5-fluoro-2'-deoxyuridine (FUDR, ***2***) are fluoropyrimidines used in the treatment of a variety of human cancers. Their mechanism of activation is shown in Figure 1.

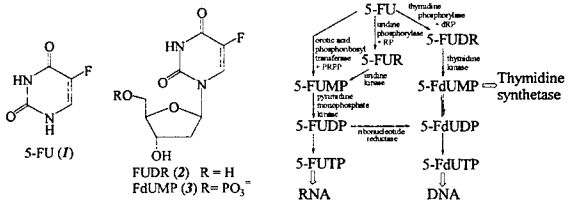


Figure 1. The structure and activation mechanism of 5-FU and FUDR.

The above activation of 5-FU and FUDR occurs in normal cells as well as tumor cells. We are modifying the structure of fluoropyrimidines through the incorporation of a trigger activation mechanism so that the new molecule could be activated site-specifically in the target tumor cells. Two types of activation mechanisms are being considered:

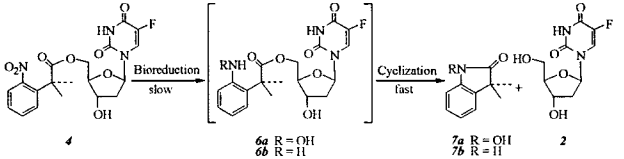
a) Bioreduction in hypoxic or oxygen-deficient tumor tissues
b) Reduction by a nitroreductase delivered to tumor cells

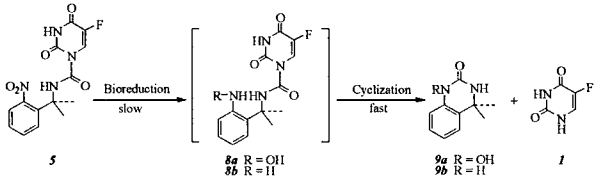
Our goal: to design and synthesize fluoropyrimidine prodrugs that could be activated by hypoxic tumor tissues or by a reductase enzyme delivered to tumor cells. These prodrugs could potentially increase the therapeutic efficacy of chemotherapy, as well as decrease its systemic toxicity.

DESIGN PRINCIPLE

Two potential prodrugs (***4, 5***) of fluoropyrimidines containing the 2-nitroaryl moiety were desinged. Their proposed activation mechanisms are shown below in Schemes 1.

Scheme 1. Proposed activation mechanisms.



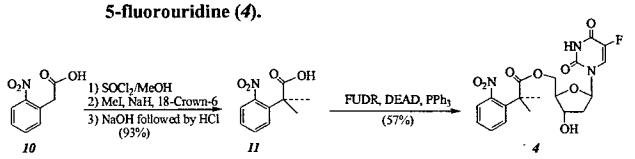


Since the bioreduction shown above is designed to occur predominantly in hypoxic tumor tissues or in tumor cells where a nitroreductase is already delivered, the compounds should achieve selective anticancer activity with minimal side effects.

SYNTHESIS

The FUDR analog, 5'-[2-(2-nitrophenyl)-2-methylpropionyl]-2'-deoxy-5-fluorouridine (***4***), was synthesized starting from 2-nitrophenylacetic acid in four steps with an overall yield of 53% (Scheme 2).

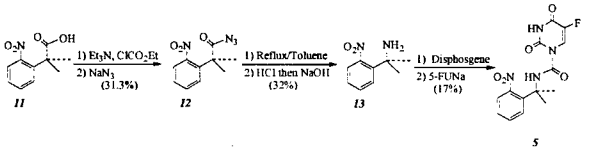
Scheme 2. Synthesis of 5'-[2-(2-nitrophenyl)-2-methylpropionyl]-2'-deoxy-5-fluorouridine (*4*).



SYNTHESIS (Cont'd)

The 5-FU analog, 1-[2-(2-nitrophenyl)-2-methylethylcarbamoyl]- 5-fluorouracil (***5***) was synthesized starting from 2-(2-nitrophenyl)-2-methylpropionic acid in six steps with an overall yield of 2% (Scheme 3).

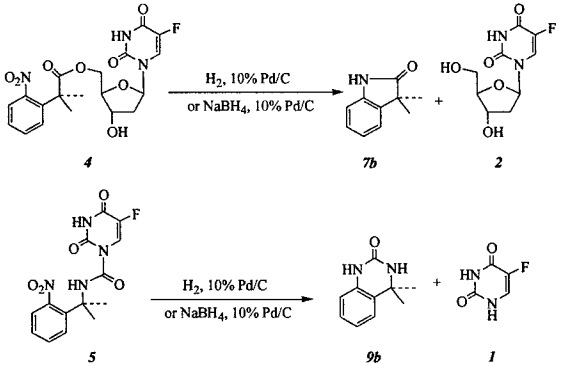
Scheme 3. Synthesis of 1-[2-(2-nitrophenyl)-2-methylethylcarbamoyl]-5-fluorouracil (*5*).



BIOMIMETIC REDUCTION

Both the FUDR and the 5-FU analogues were found to release the parent compound upon reduction of the nitro group to the corresponding amino group. The reduction conditions used were hydrogenation or sodium borohydride over 10% palladium on carbon (Scheme 4).

Scheme 4. Biomimetic reduction of *4* and *5*.



Acknowledgments

Oklahoma Center for the Advancement of Science and Technology (OCAST)
Department of Defense Prostate Cancer Research Program

DEPARTMENT OF THE ARMY
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REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y) 28 July 03

MEMORANDUM FOR Administrator, Defense Technical Information Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir, VA 22060-6218

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

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PHYLLIS M. RINEHART
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